LONGEVITY OF LIFE PART 1: DEVELOPMENT OF A BACTERIAL SPORE LIVE/DEAD ASSAY

Final Report

JPL Task 1014

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A. OBJECTIVES

Bacterial spores (endospores) are the most durable form of life, and are produced by some microorganisms during adverse environmental conditions. They remain in a dormant state and exhibit no detectable metabolism until conditions become more favorable for vegetative growth. During dormancy, endospores enjoy increased resistance to heat, UV and gamma radiation, extreme desiccation and oxidizing agents [1-3]. The objective of this work was to demonstrate a novel method for quantifying both total concentrations and the viable fractions of bacterial spores (i.e., endospores), which we call the endospore viability assay (EVA). EVA was developed at JPL for the validation of spacecraft bioburden reduction.

B. PROGRESS AND RESULTS

It was found that (1) the endospore viability assay can be correlated to traditional microbiology methods of direct microscopic enumeration and culturing methodologies, and (2) endospore viability can be determined for endospores embedded in soils, using anion chromatography. We have obtained sea-sediment samples from our colleagues at the U. Rhode Island. The breakdown is shown below:

(1) The Endospore Viability Assay (EVA) allows us to determine the fraction of the total endospores in a sample that is able to germinate. Germination, the process through which an endospore goes to again form colonies and reproduce, takes place in several stages. During the first, DPA is released from the spore into solution [1]. This is the step on which the EVA depends. That DPA is then able to bind to Tb^{3+} ions in the solution and subsequently fluoresce brightly under excitation; this fluorescence intensity is measured and correlated to a concentration of spores. Following germination, the entire sample is autoclaved to lyse the remaining spores. This frees the DPA from the spores that were unable to germinate, i.e., that were not viable. This fluorescence intensity is then measured, and a corresponding concentration is found. By knowing the amount of spores that germinated and knowing the total number of spores, it is possible to divide the two numbers and obtain a ratio that indicates the fraction of the total spores that were viable.

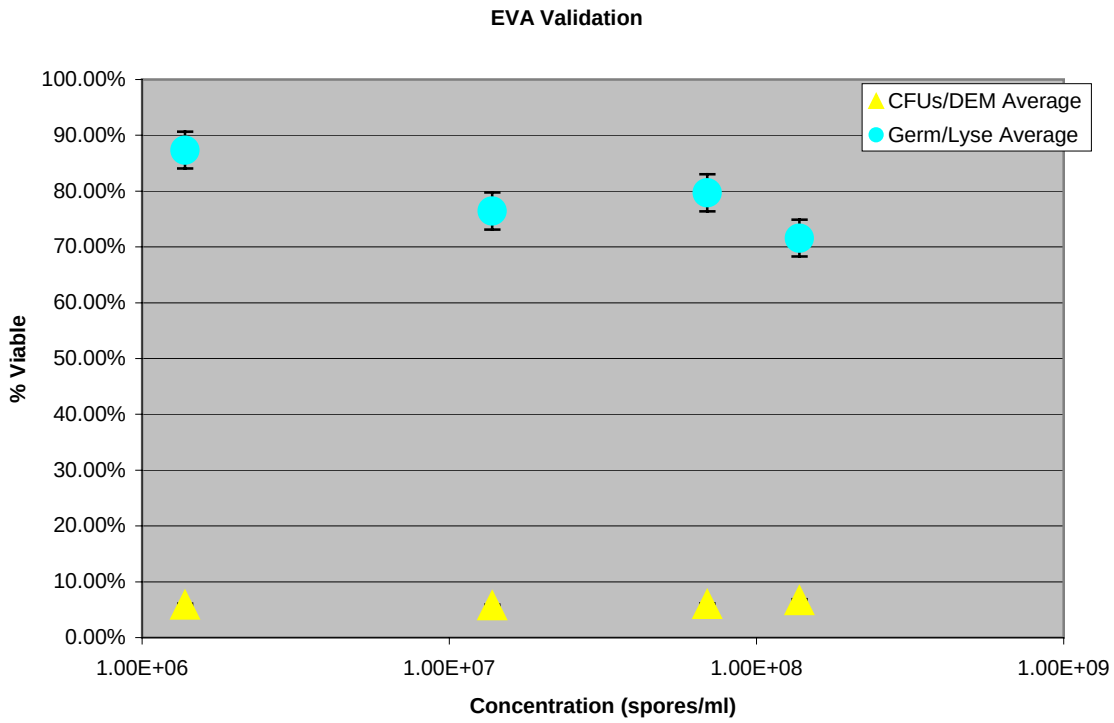

Figure 1 Correlation between % viable using the EVA compared to using standard methods of CFU formation vs. DEM. You can see that there is a large disparity between the 2 figures, illustrating the need for a quantitative EVA in sterilization procedures. Shown with error bars at +/- 1 standard error, based on 9 data points for each concentration and each series.

To validate the EVA and align it with standard methods of determining endospore viability, we worked to gather EVA data from a sample and compare it to current, "gold-standard" methods. These include using the number of colony-forming units (CFUs) to gather a count of the viable spores and using direct enumeration under a microscope (DEM) to obtain a total number. This gives a ratio that indicates the fraction viable under those conditions. This can be and was compared to the fraction viable under the EVA. This process was repeated multiple times for different spore concentrations. We have found that, though through plating spores, only around 6% give rise to colonies, a very large fraction is viable enough to undergo stage-one germination and release DPA (see Figure 1. This means that for all the assays that use simple CFU formation to check sterilization, they miss up to 80% of the spores that are capable of germinating. These spores, though they may not immediately form colonies, can still produce a vegetative cell that is able to cause damage [1]. Because of this, the EVA may be greatly useful in preventing contamination — it is over ten times more sensitive than the current method.

In order to accurately determine the concentration of spores to get a precise ratio, it was necessary to correlate fluorescence intensity to concentration, using known concentrations. A serial dilution was run, beginning with an initial, high concentration of lysed spores. Since 10 mM L-alanine was used to induce germination, and 100 μM Tb^{3+} was used to complex with DPA, both of these were in solution during the dilution, which was run at 37° C. This is the temperature at which the germination was run, as well as the temperature at which fluorescence intensities for germinated and lysed spores were measured, for it is the optimal germination temperature for *Bacillus subtilis*, the spores that were used. This dilution gave rise to an equation which, with relatively good accuracy, allowed us to correlate intensity to concentration: $y = 0.0202 * x^{.9767}$ with $R^2 = 0.9952$ (see Figure 2, y representing fluorescence intensity at 543.5

nm in counts per second (cps) and *x* representing the concentration of spores in spores per milliliter (sp/ml). Using this formula, we were able to attain precise concentrations for a given intensity and determine with more accuracy the ratio of viable-to-total spores.

Initially, microwave radiation at 140° C and constant pressure was used to lyse the spores. While this does successfully burst the thick spore coat, it also alters the Tb-DPA complex, which is undesirable. We found that the microwave radiation has destructive, catalytic effects on the Tb-DPA complex that forms in water. After being exposed to eight minutes of microwaves, the intensity of the complex is reduced to around 50% of its original intensity. After subsequent microwaves, the intensity remains the same. This is only an issue with the complex; when microwaved individually and then combined, the intensity is constant. Additionally, in water, 1 mM Tb^{3+}, which is concentrated enough to fluoresce weakly, maintains its intensity even after multiple microwaves. This data was then compared to intensities following autoclaving, where the samples were subjected to the same heat and pressures but were not exposed to the microwaves. These did not decrease after being heated; instead, they remained constant. This leads to the conclusion that the degradation of the Tb-DPA complex is catalyzed strictly by microwaves, not by heat or pressure The microwaves, which would rotate the highly polarized Tb-DPA molecules rapidly in order to raise the temperature, could generate "hot pockets" near some of the molecules where the local temperature would be much higher than the average temperature of the solution. This extreme temperature could catalyze the decomposition of the compound. Another possible explanation for this degradation is that the rapid rotation of the huge Tb-DPA molecules could lead to shearing of the molecule, where it simply breaks apart because of the huge centrifugal force [3,4]. Either hypothesis is plausible; it will require careful examination of the products of this degradation through other analytic methods to determine the process. The same effects were found using Eu^{3+}, another lanthanide that couples with DPA to fluoresce brightly, which suggests that it is not just typical of Tb^{3+} but would happen with all lanthanides when complexed with DPA.

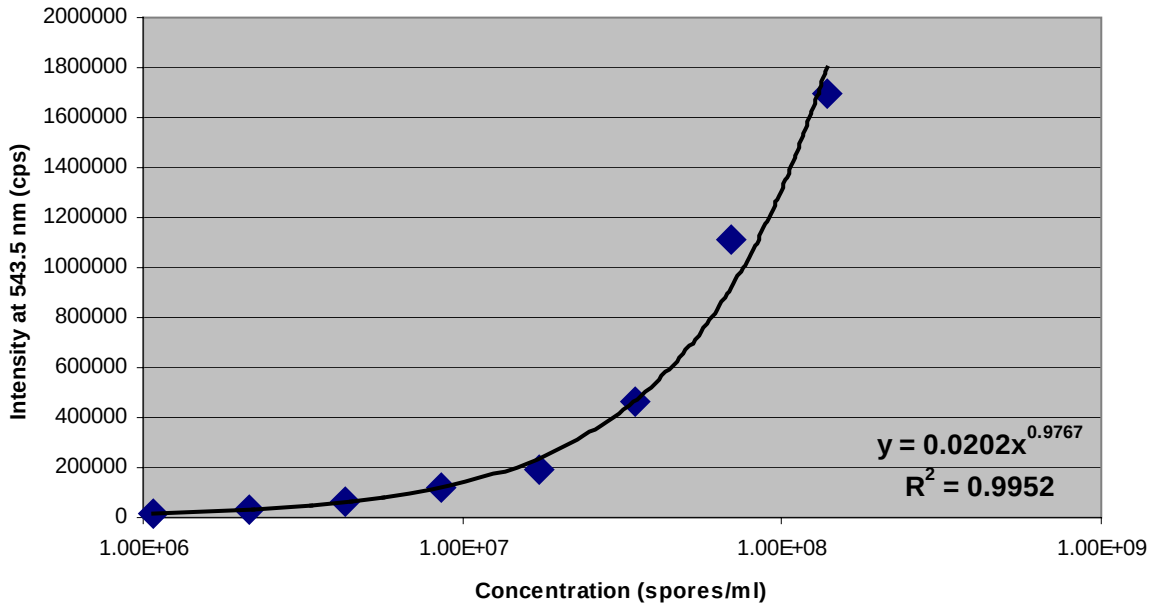

Figure 2 Established relation between released DPA from spores and intensity at 543.5 nm. Equation was determined by a Best-Fit trend line.

(2) These results came about because of our initial use of the microwave to lyse spores. Upon these discoveries, we have been using the autoclave—it is evident that in water, the autoclave has no effect upon the Tb-DPA complex. The microwave-catalyzed degradation could be responsible for a loss in sensitivity of the EVA and inconsistent results. Now, we can avoid these problems and troubleshoot other aspects. For instance, when we attempted to dope sand and soil with spores, trying to see Tb-DPA fluorescence in complex media, we found that there was too much in the sample to allow the fluorescence at 543.5 nm to stand out (see Figure 4.) We began using Ion Chromatography (IC) to quantify the DPA in complex media such as sand or soil; this allows us to study these media in terms of spores that might be present. We can see the signature of DPA in the conductance charts, and by integrating the peak, can establish a concentration. This will give us another method of quantifying viability, and will be useful when examining solutions not composed strictly of water or glycerol.

Primary validation of the EVA with standard methods has been accomplished. Further validation, using IC as a third set of data, is in order. Once this has been accomplished, we will begin to look at interesting samples such as sea sediments and ice cores. We will then be able to make conclusions about endospores in places where life was thought to be impossible, like the bottom of the ocean. Ice cores, drilled from the Antarctic, provide a reliable depth-to-age correlation. Determining viability of endospores in the ice at different depths and establishing a viability-decay curve over time would allow us to quantitatively state how long an endospore can remain viable, i.e., its maximum age. This has many implications, for if it is long enough, it could be extended to searching for life outside of Earth -- on Mars, for instance. Establishing a rapid, quantitative method for determining endospore viability, our EVA has widespread applications. From this, we would learn about nature's most primitive and resilient form of life, enhancing our knowledge immensely of the world in which we live.

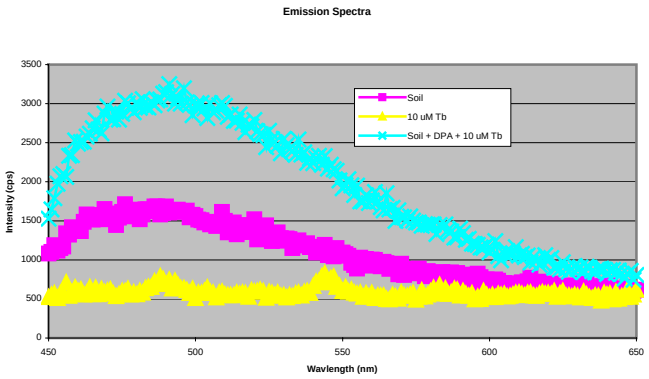

Figure 3 Rather than the characteristic Tb^{3+} emission spectrum, the emission spectra in soil have such high absorbance and background fluorescence due to other compounds in the soil that it is impossible to see that Tb, let alone Tb-DPA, are in solution. This indicates that the fluorescence method of quantifying DPA will not work in complex media.

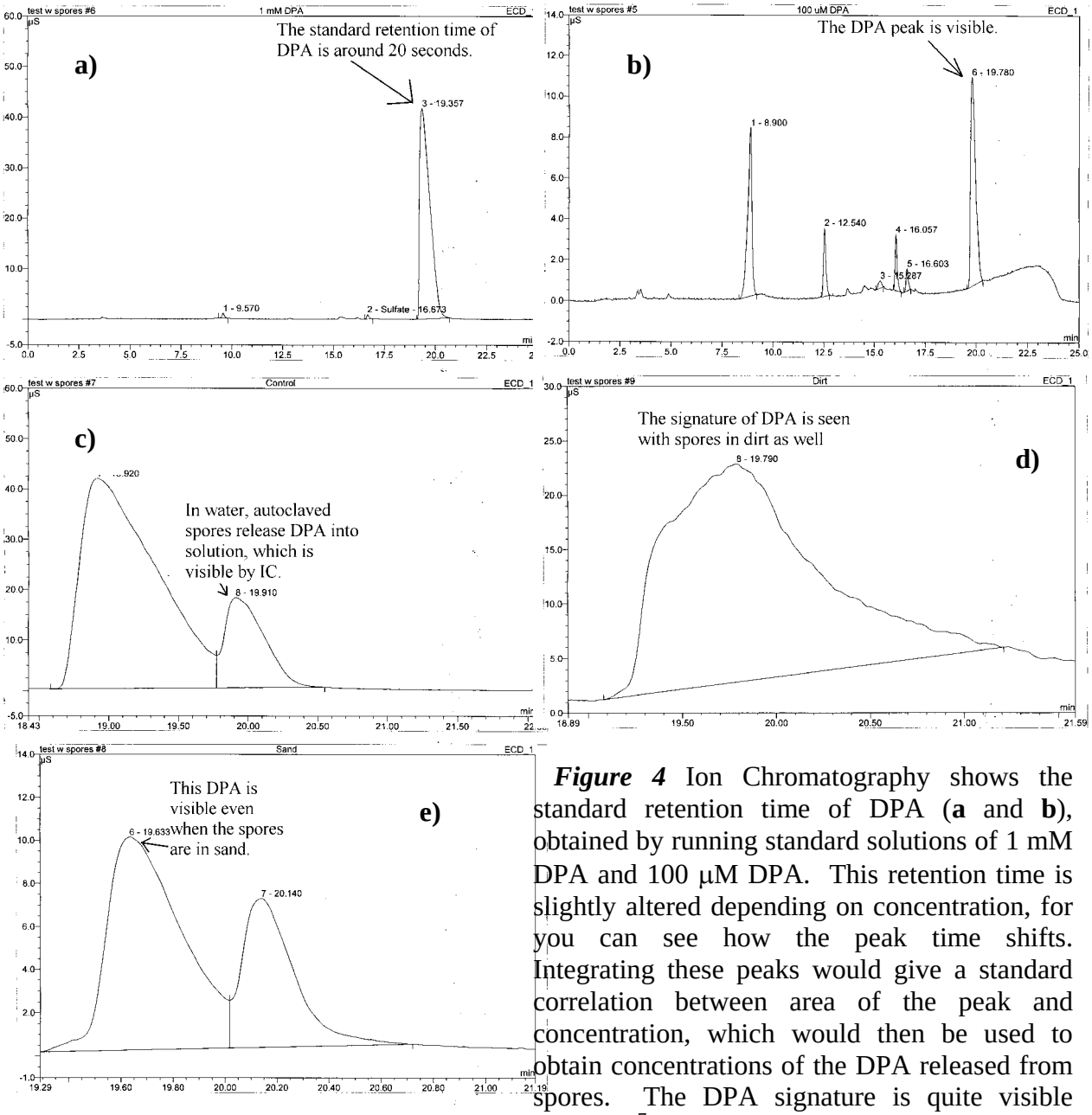

Figure 4 Ion Chromatography shows the standard retention time of DPA (**a** and **b**), obtained by running standard solutions of 1 mM DPA and 100 μM DPA. This retention time is slightly altered depending on concentration, for you can see how the peak time shifts. Integrating these peaks would give a standard correlation between area of the peak and concentration, which would then be used to obtain concentrations of the DPA released from spores. The DPA signature is quite visible when 10^7 sp/ml are lysed in water (**c**) and complex media such as dirt and sand (**d** and **e**, respectively).

C. SIGNIFICANCE OF RESULTS

This task developed an endospore viability assay capable of determining the viable fraction of bacterial spores in environmental samples. This novel assay will be used in future studies to determine the viability of the toughest form of life, the endospore, in the most extreme environments on Earth. In addition, we will develop instrumentation for automated EVA measurements with an eye on searching for the chemical signature of endospores in Martian polar ice caps.

D. FINANCIAL STATUS

The total funding for this task was $185,100 all of which has been expended.

E. PERSONNEL

No other JPL personnel were involved.

F. PUBLICATIONS

We have two peer-reviewed publications in preparation on (1) the validation of the endospore viability assay, and (2) the microwave-induced degradation of DPA in the presence of lanthanides.

G. REFERENCES

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[2] Jones, Guilford II; Vullev, Valentine I. "Medium effects on the photophysical properties of terbium (III) complexes with pyridine-2,6-dicarboxylate." *Photochemical and Photobiological Sciences* 2002, 1:925-933.

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[5] Nicholson, Wayne L; Setlow, Peter. 1990. "Sporulation, germination, and outgrowth", p. 391-450. *In* C. R. Harwood and S. M. Cutting (ed.), Molecular biological methods for *Bacillus*. John Wiley & Sons, Sussex, England.